0273-2289/91/2703—0195$2.00

Liquid Fluidized Bed Starter Culture of *Trichoderma reesei* for Cellulase Production

R. P. TENGERDY,*[,1] W. H. RHO,[1] AND A. MOHAGHEGHI[2]

[1]Department of Microbiology, Colorado State University, Fort Collins, CO 80523; and [2]Biotechnology Branch, Solar Energy Research Institute, 1617 Cole Blvd., Golden, CO 80401

Received February 13, 1990; Accepted April 5, 1990

ABSTRACT

A starter culture of *Trichoderma reesei* (Rut-C30) prepared in a liquid fluidized bed reactor (LFBR) gave better growth and greater cellulase production in submerged fermentation than a conventional shake flask inoculum. The LFBR starter was prepared by first coating *T. reesei* spores to 0.25 mm size corncob ($1.0\times10^8 g^{-1}$) in a medium containing 1.0% corncob, 0.5 gL^{-1} xylose and 0.1 gL^{-1} lactose in a balanced salt solution, then fluidizing the particles in the LFBR for 36 h to allow germination of the spores, and covering the particles with an approx 30 μm thick biofilm. This biofilm that developed in constant adherence to the lignocellulosic carrier, apparently became well adapted to grow rapidly on insoluble cellulose substrates (Solca Floc), and had the enzymes of the cellulase complex induced for increased cellulase production.

The LFBR starter used in a stirred tank reactor (STR) gave 15 gL^{-1} biomass production and 6.5 IU mL^{-1} overall cellulase activity with a volumetric productivity of 64 IU $L^{-1}h^{-1}$ in a 5 d fermentation, compared with a 7 d shake flask inoculum that gave 11 gL^{-1} biomass and 3.2 IU mL^{-1} cellulase activity, with a volumetric productivity of 31 IU $L^{-1}h^{-1}$. The LFBR starter culture retained its viability in dry storage for 6–9 mo.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Cellulase, production; liquid fluidized bed reactor (LFBR); *Trichoderma reesei,* in cellulose fermentation; cellulose, as substrate for fermentation; corn cob, as carrier in LFBR.

INTRODUCTION

The use of appropriate starter cultures and inoculation procedures greatly influences cellulase production by cellulolytic fungi both in submerged fermentation (SF) (*1*) and solid substrate fermentation (SSF) (*2*). A vigorous fungal growth, resulting in high biomass concentration, subsequently leads to high cellulase production. The preinduction of the enzymes of the cellulase system in the starter culture may lead to greater productivity of cellulases. An optimal starter should achieve both goals, promoting rapid, active growth and greater cellulase production.

Traditionally, spore suspensions or mycelial cultures are employed as inocula in fungal fermentations. The drawback of the spore inoculum is the long lag period in the fermentation, resulting from slow spore germination and enzyme induction. The mycelial inoculum preparation requires a long time and several scale up steps (*1*). A carrier attached biofilm as starter culture appears to have advantages over the traditional inocula. In previous research, spores of *Chaetomium cellulolyticum* and *Trichoderma reesei* were germinated on moist furfural bran particles in a gas fluidized bed reactor (GFBR), to be used as starter in SSF of lignocellulose (*3*). An active fungal biofilm of *T. reesei* on corn cob was prepared in a liquid fluidized bed reactor (LFBR) and used successfully as starter in SSF of lignocellulose (*4*). The GFBR produced a semi-dry starter, easy to store and ready to use, but the LFBR starter contained more biomass and produced better growth in SSF.

The carrier attached biofilm starters appear to have an advantage over traditional starters, mainly because of the conditions of fungal growth in a fluidized bed reactor. The fungal mycelia in an LFBR develop while constantly attached to the lignocellulose carrier surface, thus adapted to growth on solid cellulosic particles. The surface attachment may also contribute to the induction of enzymes for cellulose degradation. Because of the high packing density of spores and developing hyphae on the carrier, higher biomass concentration can be reached on the drained carrier than in a liquid culture, and viability is retained longer on the protective surface of the carrier, making possible the storage of the starter for protracted use. Starter culture production in the LFBR is a single step operation with simple separation of biofilm coated particles by draining.

This paper describes the preparation of an active starter culture of *T. reesei* (RUT-C30) on corncob in the LFBR, and compares its use with a conventional mycelial inoculum in an advanced submerged fermentation system for cellulase production (*1*).

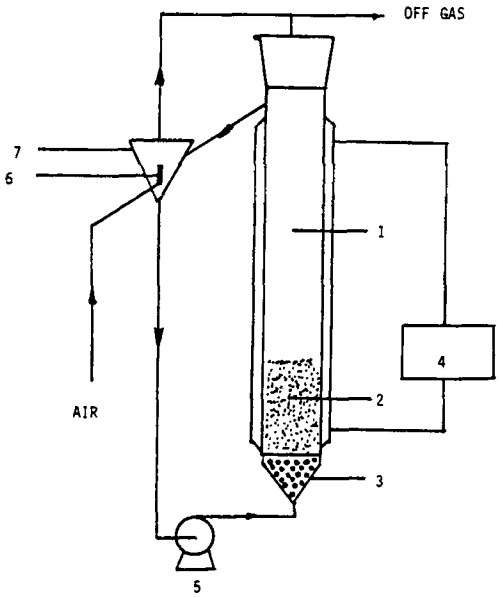

Fig. 1. Liquid fluidized bed reactor: 1. Glass reactor; 2. Substrate (corncob); 3. Glass beads; 4. Water bath; 5. Recirculation pump; 6. Air sparger; 7. Liquid-solid separator.

MATERIALS AND METHODS

Stock Culture and Starter Cultures

Trichoderma reesei (RUT-C30) spores, suspended in 10% glycerol, were preserved at −70°C as stock culture (*1*). For mass spore production, the stock culture was grown on potato dextrose agar in 500 mL Roux bottles with the addition of furfural bran (5 gL^{-1}) at 28°C in daylight to enhance sporulation. The spores were harvested by washing with water containing 0.01% Tween 80, then concentrated to 1.0×10^8 spores mL^{-1} and stored at 4°C. This spore suspension was used for preparing starter cultures.

A three stage mycelial inoculum was prepared as described earlier (*1*). Briefly, 1 mL spore suspension was inoculated into 25 mL medium in a 100 mL flask, incubated for 2 d, then transferred successively into 50 mL medium in 250 mL flask for 2 d and 250 mL medium in 1000 mL flask for 3 d for a total incubation time of 7 d on a rotary shaker at 150 rpm, 28°C. The first stage medium contained 1.0% glucose, the second 1.0% Solka Floc BW200 (James River Co., NH), the third 5.0% Solka Floc as carbon source. All media contained 10 gL^{-1} corn steep liquor, 1.4 gL^{-1} $(NH_4)_2SO_4$, 0.4 gL^{-1} $CaCl_2 \cdot 2H_2O$, 0.3 gL^{-1} $MgSO_4 \cdot 7H_2O$, 1.56 mgL^{-1} $MnSO_4 \cdot H_2O$, 1.4 mgL^{-1} $ZnSO_4 \cdot 7H_2O$, 2.0 mgL^{-1} $CoCl_2$, 5.0 mgL^{-1} $FeSO_4 \cdot 7H_2O$, and 0.2 mL L^{-1} Tween 80. The pH was 4.8.

LFBR starter culture was prepared in the reactor shown in Fig. 1. The operation of the LFBR for microbial starter culture production was de-

scribed in detail elsewhere (*3–6*). The reactor was a jacketed glass column, 3.5 cm inside diameter, 1 m in height, with a working vol of 1.5 L. Ground corncob #4060 (Grit-O-Cob, The Andersons, Cob Division, Maumee, OH, 40–60 mesh, 0.25 mm diameter and 500 kg/m^3 bulk density), was selected as carrier and carbon source because of its favorable physical properties for fluidization (*4*). The sterilized corncob was coated with 1.0×10^8 spores g^{-1} DW in a medium containing 10 gL^{-1} corncob, 0.5 gL^{-1} xylose, 0.1 gL^{-1} lactose, 10 gL^{-1} corn steep liquor, and the same salt mixture as in the mycelial inoculum, pH 4.8. One and a half liter of the coated spore suspension was loaded into the sterilized LFBR. The temperature in the LFBR was maintained at 28°C during operation. The spore suspension was fluidized initially at 0.1 cm sec^{-1} fluidization velocity, then the velocity was gradually increased to 0.25 cm sec^{-1} as the biofilm of germinating spores and young hyphae developed, to prevent aggregation of particles. The liquid medium was recirculated in the LFBR with a peristaltic pump. The entering upflow stream was passed through a layer of 3 mm diameter glass balls at the bottom of the reactor to distribute the liquid evenly. The liquid exited through an overflow into a conical separator to settle out free mycelia and light corncob particles. To avoid air bubbles forming and upsetting the flow pattern in the LFBR, the medium was aerated in the separator, by sparging air through the liquid at the upper part of the separator at 40 Lh^{-1}. After 36 h, the liquid was drained, and the wet biofilm coated particles were used immediately as starter culture, or air dried and stored for viability testing. In the experiments reported here, the LFBR was operated in a batch mode, but continuous operation is possible if the biofilm coated lighter particles are continuously withdrawn in the overflow and spore coated heavier particles are fed at the bottom of the reactor (*3*).

Submerged Fermentation in Stirred Tank Reactor (STR)

Bench top fermenters (B. Braun, Biostat Model V, Allentown, PA) (5 L) were used for cellulase production with a working vol of 2 L. The medium contained 50 g Solca Floc or a mixture of 30 g xylose and 20 g Solca Floc as carbon source, 10 g corn steep liquor, 11.7 g $(NH_4)_2SO_4$, 0.8 g $CaCl_2 \cdot 2H_2O$, 0.6 g $MgSO_4 \cdot 7H_2O$, trace elements as in the inoculum medium, and 0.2 mL Tween 80/L. The pH was maintained at 4.8 and temperature at 28°C. Dissolved oxygen was controlled between 20–100% of saturation value by varying the air flow rate or pure O_2 flow rate and agitation rate. Foaming was controlled by the addition of 1:20 diluted Antifoam B (Sigma Chem Co., St. Louis, MO). Samples were taken at 24-h intervals.

Analyses

Dry wt (DW) of samples from the LFBR and STR was determined by centrifugation and drying overnight at 105°C. Fungal protein was deter-

Table 1
Effect of Starter Cultures
on Cellulase Production by Submerged Fermentation

	Starter culture					
	LFBR			Shake flask		
Carbon Source	FPA	VP	Biomass Protein	FPA	VP	Biomass Protein
Solka Floc 50 gL^{-1}	6.45±1.1a	64.0±10.1a	15±2.1a	3.21±1.0a	30.9±10 1a	11±0.9a
Xylose 30/Solka Floc 20 (gL^{-1})	4.63±0.9b	38.6±9 0b	16±1.2a	2.74±0.8a	22.8±8 0a	13±1.1a

FPA = filter paper activity [IU mL^{-1}]; VP = volumetric cellulase productivity [IU $L^{-1}h^{-1}$]; Biomass protein [gL^{-1}]; 5 d fermentation in a 5L stirred tank reactor (STR) at 28°C, pH 4.8, inoculum size 10% w/w of both starters. The data are means of 3 STR runs per treatment. The differences between the LFBR and shake flask inoculum were significant ($p \leq 0.01$) in all three categories (horizontal columns). Significant differences in the carbon source effect are indicated by different small case letters in the vertical columns ($p \leq 0.05$).

mined as the trichloroacetic acid precipitable Kjeldahl nitrogen of washed samples (*7*). Biomass was estimated by multiplying the protein value by 2.7, since the average protein content of *T. reesei* was 37% (*1*). Cellulose content was estimated as the difference between the dry wt of sample and the estimated dry wt of fungal biomass. Cellulase activity was measured as filter paper activity by the standard IUPAC method (*8*), and expressed in IUg^{-1} DW. Reducing sugars were measured by the dinitrosalicilic acid (DNSA) method (*9*). Beta-glucosidase activity was expressed as μmol *p*-nitrophenyl released/min from *p*-nitrophenyl-beta-D-glucopyranoside at 50°C (*10*). All assays were run in duplicate. All experiments reported here were repeated several times. The data in Table 1 are means of 3 STR runs/treatment, evaluated statistically by analysis of variance. In the figures, the most representative single runs in the LFBR or STR are shown.

RESULTS AND DISCUSSION

Fungal Growth in the LFBR

A representative typical growth curve of *T. reesei* in the LFBR is shown in Fig. 2. In the first 36 h, the growth was nearly linear and consisted of a thickening biofilm on the surface of the corncob. At 36 h, the thickness of the biofilm was about 30 μm. This was estimated from scanning electron micrographs. Beyond 36 h, the biofilm thickness did not increase significantly, but free mycelia appeared in the liquid, and particles tended to stick together. The drained 36-h starter culture at 70% moisture content contained in average 32.5 mg g^{-1} (wet wt) fungal biomass, compared to 40 mg mL^{-1} in the 7 d mycelial starter. This means that the 36 h LFBR

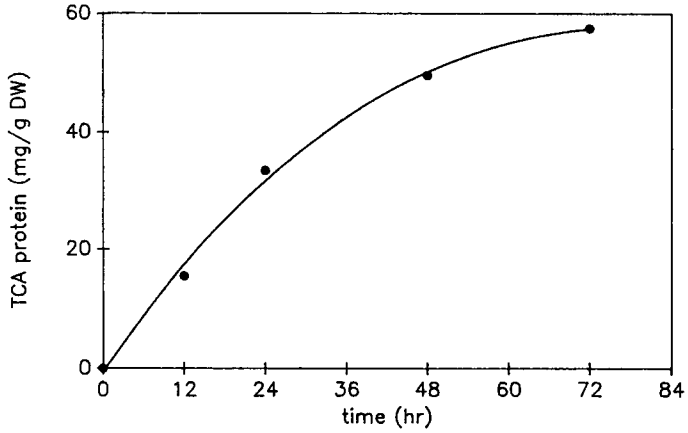

Fig. 2. Growth of *T. reesei* (RUT-C30) in the LFBR. Inoculum, 1.0×10^8 spores g^{-1} DW corncob; temperature 28°C; fluidization velocity, 0.10 cm sec^{-1} 0–12 h, 0.15 cm sec^{-1} 12–24 h, 0.25 cm sec^{-1} 12–18 h, 0.35 cm sec^{-1} 18–72 h. TCA protein: tricholoracetic acid precipitated protein assayed as Kjeldahl nitrogen.

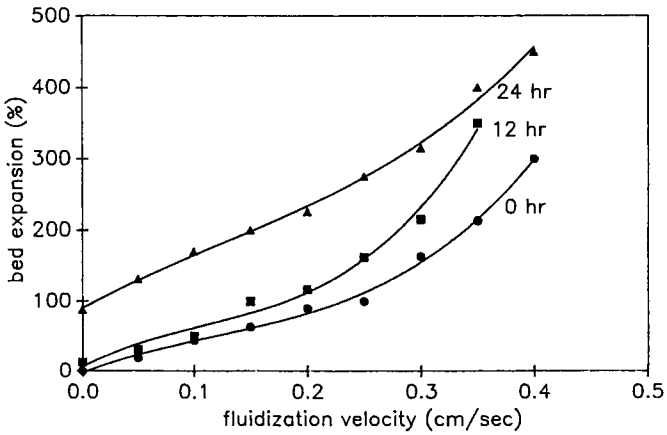

Fig. 3. Bed expansion in the LFBR. Conditions are the same as in Fig. 2.

starter contained almost as much biomass as the 7 d mycelial starter. The high biomass concentration in the LFBR was achieved by the initial high concentration of spores on the surface of the corncob (about 250 times more spores were used in the LFBR than in the shake flask for equivalent biomass production), the rapid germination and growth of surface bound mycelia, and the high packing density (vol of biomass/vol of carrier) of biomass on the carrier. One advantage of the LFBR is this ability to concentrate and convert an inactive form of the fungus (spores) into an active biomass.

The bed expansion is an important indicator of biofilm development in the LFBR, as shown in Fig. 3. As biofilm grew, the density of the loaded particles decreased, and they started to rise toward the top. From 12 h on, the bed expansion became sensitive to increasing fluidization velocity. At 12-h and 24 h-fluidization, beyond 0.4 cm s^{-1} velocity, the loaded parti-

cles were washed out from the reactor. This feature may be used in designing a continuous starter culture production. At a carefully selected point of bed expansion, biofilm development, time, and fluidization velocity, the upward moving biofilm loaded particles may be sorted out in the overflow of the reactor.

In batch operation, the fluidization velocity was changed according to the changing behavior of loaded particles, as revealed in the bed expansion curve. The LFBR was started at 0.1 cm s^{-1} fluidization velocity for 12 h, continued with 0.15 cm s^{-1} in the next 6 h, and finished with 0.25 cm s^{-1} in the last 18 h. The LFBR was stopped at 36 h, when biomass formation reached 75% of the maximum growth potential, and biofilm thickness reached its maximum. Beyond this point, detached mycelia disturbed fluidization, clogged the reactor, and caused channel formation. The loaded particles were drained from the reactor and used directly as a starter culture for cellulase production in the STR.

LFBR Starter vs Shake Flask Starter for Cellulase Production in STR

The comparison of the two starters is shown in Table 1 and Fig. 4. Both starters were used in the STR at the 10% (w/w) level, containing comparable amounts of fungal biomass, as shown above. The LFBR inoculum gave consistently higher average cellulase activity (6.5 IU mL^{-1}) in 5-d fermentation than the shake flask inoculum (3.2 IU mL^{-1}). The volumetric productivity of cellulase was also correspondingly higher (64 IU $L^{-1}h^{-1}$ vs 31 IU $L^{-1}h^{-1}$). With both starters, Solca Floc as sole carbon source consistently gave higher cellulase activity and productivity than a mixture of Solca Floc and xylose, but in the presence of xylose, fungal growth was slightly greater, corroborating our earlier findings (*1*).

Although the LFBR starter gave significantly higher biomass productivity than the shake flask starter (Table 1, Fig. 4), this difference or the slight differences in substrate conversion rates were not enough to explain the large increase in cellulase productivity. It appeared that the cellulase system was better induced in the LFBR starter. To investigate this point, the effect of soluble inducers, lactose and salicin, was tested in LFBR and shake flask starters. Lactose and salicin both induced the LFBR starter as can be seen in Fig. 5, resulting in better cellulase production in the STR. The same inducers had no effect in the shake flask starter. Since an LFBR starter without inducer (control in Fig. 5) still gave higher cellulase production than the shake flask starter, it may be assumed that surface growth on the insoluble carrier may also contribute to induction.

The LFBR starter represents a new concept in microbial starter culture production. In traditional microbial starters, the goal is the production of a vigorously growing active biomass. In the LFBR, emphasis is on the activation of large number of surface bound spores by rapid germination, and induction of enzyme systems. Extensive mycelial development in the

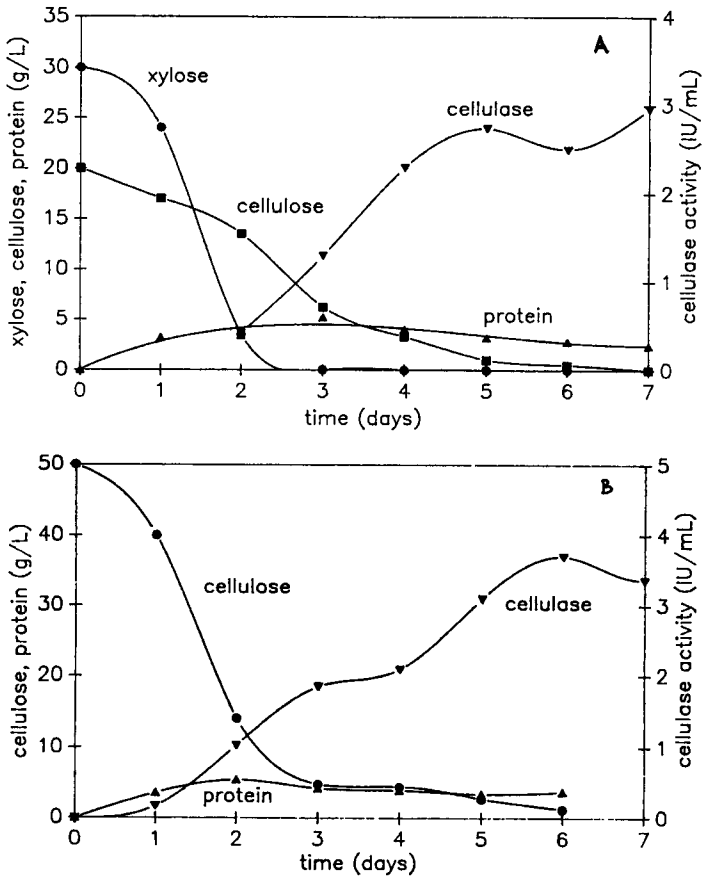

Fig. 4. Comparison of LFBR and shake flask starter cultures for growth and cellulase production in STR. A, 3% xylose, 2% Solka Floc medium, 10% shake flask inoculum; B, 5% Solka Floc medium, 10% shake flask inoculum;

LFBR is not expected, growth is only initiated for rapid continuation in the STR.

A practical advantage of this type of starter is that at this early developmental stage the activated fungus, that is well protected on the carrier surface or inside its pores and cavities, may retain its viability for a long time for a protracted use. In our experience, air dried, sealed *T. reesei* LFBR starters retained their viability for 6–9 mo. A lactic acid starter culture, prepared in the LFBR on corncob carrier in this laboratory, also had extended storability, and was used as a prepackaged silage additive (*5,6*).

CONCLUSIONS

LFBR starter cultures of *T. reesei* (Rut-C30) gave superior cellulase production in STR fermentations compared with shake flask starter cultures. The LFBR allowed surface concentration of large number of spores

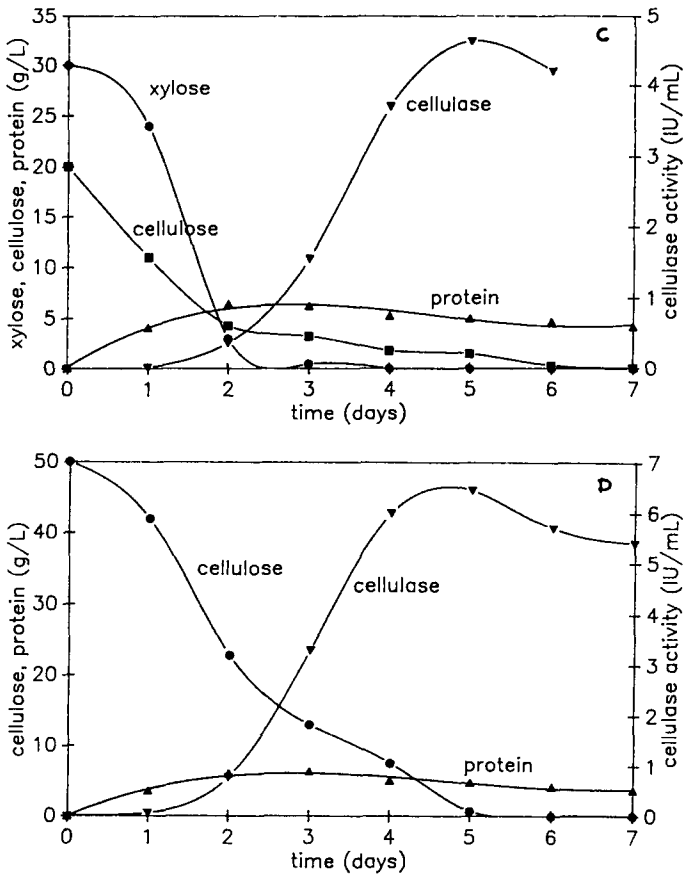

Fig. 4 (*cont'd*). C, 3% xylose, 2% Solka Floc medium, 10% LFBR inoculum; D, 5% Solka Floc medium, 10% LFBR inoculum. Temperature 28°C, pH 4.8.

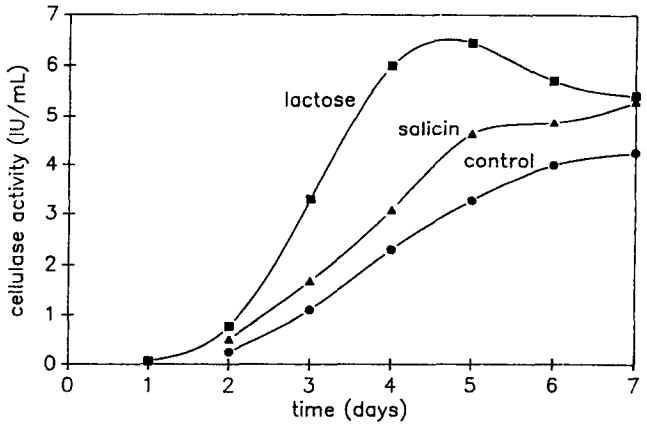

Fig. 5. Effect of inducers on cellulase productivity. The inducers, 0.1 gL^{-1} lactose or salicin, were present in the LFBR during starter culture production. STR fermentation: C-source, 50 gL^{-1} Solka Floc, 10% LFBR inoculum, 28°C, pH 4.8, 5 d.

and their rapid germination into an active biomass with an induced cellulase system. The adaptation to growth on solid surfaces, and the efficient induction of the cellulase system are the most probable reasons for the superior performance of the LFBR starters. The rapidity and simplicity of production, the storability and easy dosage of dried starters are advantages for practical applications.

ACKNOWLEDGMENTS

This research was supported by Solar Energy Research Institute (SERI) subcontract XK-7-07031-7 to Colorado State University, and by NSF grants INT-8500384, INT-8722686, and CBT-8502125. The experimental work was partially conducted at SERI, with the generous help of personnel and facilities of the Biotechnology Branch.

REFERENCES

1. Mohaghegi, A., Grohmann, K., Wyman, C. E. (1988), *Appl. Biochem. Biotechnol.* **17,** 266.
2. Tengerdy, R. P. (1985), *Trends Biotechnol.* **3,** 96.
3. Tengerdy, R. P., Mihaltz, P., Adisasmito, S., Karim, M. N. (1987), *Proc. Fourth European Congress of Biotechnology,* Amsterdam, p. 63.
4. Adisasmito, S., Mihaltz, P., Karim, M. N., Tengerdy, R. P. (1987), *Biotechnol. Techniques* **1,** 175.
5. Kemp, T. (1989), M. S. thesis, Colorado State Univ., Ft. Collins, CO.
6. Barreto, T., Kemp, T., Karim, M. N., Adisasmito, S., and Tengerdy, R. P. (1987), "Optimization of *Lactobacillus plantarum* fermentation: batch and liquid fluidized bed operations," Frontiers of Bioprocessing Conference, National Bureau of Standards, Boulder, CO, June 28—July 2.
7. Brayton, S. (1985), "A practical Kjeldahl-nitrogen method," Hach Co., Loveland, Colorado.
8. IUPAC, (1984), "Recommendations on the measurement of cellulase activities, prepared for International Union of Pure and Applied Chemistry, Commission on Biotechnology."
9. Miller, G. L. (1959), *Anal. Chem.* **31,** 426.
10. Sternberg, D. (1976), *Appl. Environ. Microbiol.* **31,** 648.